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Target of Rapamycin (TOR) in Nutrient Signaling and Growth Control

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ABSTRACT TOR (**T**arget **O**f **R**apamycin) is a highly conserved protein kinase that is important in both fundamental and clinical biology. In fundamental biology, TOR is a nutrient-sensitive, central controller of cell growth and aging. In clinical biology, TOR is implicated in many diseases and is the target of the drug rapamycin used in three different therapeutic areas. The yeast *Saccharomyces cerevisiae* has played a prominent role in both the discovery of TOR and the elucidation of its function. Here we review the TOR signaling network in *S. cerevisiae*.

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THE contributors to this GENETICS set of reviews were asked to focus on the developments in their field since 1991, the year the last yeast monographs were published. Coincidentally, **T**arget **O**f **R**apamycin (TOR) was discovered in 1991. We thus have the whole TOR story to tell, from the beginning, in a review that marks the 20th anniversary of TOR. As we review TOR signaling in *Saccharomyces cerevisiae*, the reader is referred to other reviews for descriptions of TOR in other organisms (Wullschleger *et al.* 2006; Polak and Hall 2009; Soulard *et al.* 2009; Caron *et al.* 2010; Kim and Guan 2011; Zoncu *et al.* 2011).

The story of the TOR-signaling network begins with a remarkable drug, rapamycin (Abraham and Wiederrecht 1996; Benjamin *et al*. 2011). Rapamycin is a lipophilic macrolide and a natural secondary metabolite produced by *Streptomyces hygroscopicus*, a bacterium isolated from a soil sample collected in Rapa-Nui (Easter Island) in 1965—hence the name rapamycin. Rapamycin was originally purified in the early 1970s as an antifungal agent. Although it effectively inhibits fungi, it was discarded as an antifungal agent because of its then undesirable immunosuppressive side effects. Years later, it was "rediscovered" as a T-cell inhibitor and as an immunosuppressant for the treatment of allograft rejection. Preclinical studies subsequently showed that rapamycin and its derivatives, CCI-779 (Wyeth-Ayerst) and RAD001 (Novartis), also strongly inhibit the proliferation of tumor cells. Rapamycin received clinical approval in 1999 for use in the prevention of organ rejection in kidney transplant patients, and additional applications as an immunosuppressive agent have since been developed. CCI-779 (Torisel) and RAD001 (Afinitor) were approved in 2007 and 2009, respectively, for treatment of advanced kidney cancer. Rapamycin is effective against tumors because it blocks the growth of tumor cells directly and because of the indirect effect of preventing the growth of new blood vessels (angiogenesis) that supply oxygen and nutrients to the tumor cells (Guba *et al.* 2002). Finally, rapamycin-eluting stents prevent restenosis after angioplasty. Thus, rapamycin has clinical applications in three major therapeutic areas: organ transplantation, cancer, and coronary artery disease. What do fungi and the seemingly very different conditions of transplant rejection, cancer, and restenosis have in common in their underlying biology such that all can be treated with the same drug? All three conditions (and the spread of pathogenic fungi) are due to ectopic or otherwise undesirable cell growth, suggesting that the molecular target of rapamycin is a central controller of cell growth. TOR is indeed dedicated to controlling cell growth, but what is this target and how does it control cell growth?

The Early Days

Studies to identify the cellular target of rapamycin and to elucidate the drug's mode of action were initiated in the late 1980s by several groups working with yeast (Heitman *et al.* 1991a; Cafferkey *et al.* 1993; Kunz *et al.* 1993) and mammalian cells (Brown *et al.* 1994; Chiu *et al.* 1994; Sabatini *et al.* 1994; Sabers *et al.* 1995). At that time, rapamycin was known to inhibit the vertebrate immune system by blocking a signaling pathway in helper T cells that mediates cell cycle (G1) progression in response to the lymphokine IL-2. However, the molecular mode of action of the drug was not known other than it possibly involved binding and inhibiting the cytosolic peptidyl-prolyl *cis-trans* isomerase FKBP12 (FK506-binding protein 12), also known as an immunophilin (Schreiber 1991). Furthermore, the observation that rapamycin inhibited cell cycle progression in yeast as in mammalian cells suggested that the molecular target was conserved from yeast to vertebrates and that yeast cells could thus be exploited to identify the target of rapamycin (Heitman *et al.* 1991a). It should be noted that the early

researchers were interested not only in understanding rapamycin's mechanism of action, but also in using rapamycin as a probe to identify novel proliferation-controlling signaling pathways (Kunz and Hall 1993). In the late 1980s, significantly less was known about signaling pathways than today; indeed, few and only incomplete pathways were known.

The early studies in yeast first focused on identifying an FKBP (FK506-binding protein) (Heitman *et al.* 1991b; Koltin *et al.* 1991; Tanida *et al.* 1991; Wiederrecht *et al.* 1991). FKBP12 had previously been identified in mammalian cell extracts as a rapamycin (and FK506)-binding protein. Yeast FKBP was purified to homogeneity using an FK506 column and partially sequenced. The protein sequence information was used to design degenerate oligonucleotides that were then used to isolate the FKBP-encoding gene *FPR1* (Heitman *et al.* 1991b). The predicted amino acid sequence of yeast Fpr1 was 54% identical to that of the concurrently characterized human FKBP12, providing further support that the mode of action of rapamycin was conserved from yeast to humans. Curiously, disruption of the FKBP gene in yeast (*FPR1*) revealed that FKBP is not essential for growth (Heitman *et al.* 1991b; Koltin *et al.* 1991; Tanida *et al.* 1991; Wiederrecht *et al.* 1991). Additional FKBPs and cyclophilins (also an immunophilin and proline isomerase) were subsequently discovered and cloned, and again single and multiple disruptions were constructed without consequential loss of viability (Heitman *et al.* 1991b, 1992; Davis *et al.* 1992; Kunz and Hall 1993; Dolinski *et al.* 1997). The finding that *FPR1* disruption did not affect viability was paradoxical because FKBP was believed to be the *in vivo* binding protein/target for the toxic effect of rapamycin. Why did inhibition of FKBP by rapamycin block growth whereas inhibition of FKBP by disruption of the *FPR1* gene have no effect on growth? The subsequent finding that an *FPR1* disruption confers rapamycin resistance (Heitman *et al.* 1991a,b), combined with the observation that some drug analogs are not immunosuppressive despite being able to bind and inhibit FKBP12 proline isomerase (Schreiber 1991), provided the answer to the above question and led to the well-established model of immunosuppressive drug action: an immunophilin-drug complex (*e.g.*, FKBP-rapamycin) gains a new toxic activity that acts on another target. In other words, FKBP is only a cofactor or receptor required by the drug to act on something else; FKBP itself is not the target required for viability. This mode of drug action also applies to the well-known immunosuppressants cyclosporin A and FK506 (from cyclophilin–cyclosporin A and FKBP–FK506 complexes) and is conserved from yeast to humans (Schreiber 1991). These early studies in yeast were the first of many that have since contributed to an understanding of rapamycin action and TOR signaling even in mammalian cells (Crespo and Hall 2002), illustrating that a model organism such as yeast is valuable in both basic and biomedical research.

To identify the target of the FKBP–rapamycin complex, rapamycin-resistant yeast mutants were selected (Heitman *et al.* 1991a; Cafferkey *et al.* 1993). As expected, *fpr1* mutants defective in FKBP were recovered, but also obtained were mutants altered in either one of two novel genes termed *TOR1* and *TOR2*. The *fpr1* mutations were common and recessive. Interestingly, the *TOR1* and *TOR2* mutations were rare and dominant. The *TOR1* and *TOR2* genes were cloned, on the basis of the dominant rapamycin-resistance phenotype of the mutant alleles, and sequenced (Cafferkey *et al.* 1993; Kunz *et al.* 1993; Helliwell *et al.* 1994). Both TOR1 and TOR2 proteins are 282 kDa in size (2470 and 2474 amino acids, respectively) and 67% identical. TOR1 and TOR2 are also the founding members of the PI kinase-related protein kinase (PIKK) family of atypical Ser/Thr-specific kinases (other members include TEL1, ATM, DNA-PK, and MEC1) (Keith and Schreiber 1995). Although the catalytic domain of all members of this protein kinase family resembles the catalytic domain of lipid kinases (PI3K and PI4K), no PIKK family member has lipid kinase activity, and the significance of the resemblance to lipid kinases is unknown. Two reports in 1995—before TOR was shown to be a protein kinase—claimed that yeast and mammalian TOR had lipid kinase (PI4K) activity, but these findings were never confirmed and are now thought to have been due to a contaminating PI4K. Disruption of *TOR1* and *TOR2* in combination caused a growth arrest similar to that caused by rapamycin treatment, suggesting that TOR1 and TOR2 are indeed the targets of FKBP–rapamycin and that the FKBP–rapamycin complex inhibits TOR activity (Kunz *et al.* 1993). It was subsequently demonstrated that the FKBP–rapamycin complex binds directly to TOR1 and TOR2 (Stan *et al.* 1994; Lorenz and Heitman 1995; Zheng *et al.* 1995) and that TOR is widely conserved both structurally and as the target of FKBP–rapamycin (Schmelzle and Hall 2000). However, *S. cerevisiae* is unusual in having two *TOR* genes whereas almost all other eukaryotes, including plants, worms, flies, and mammals, have a single *TOR* gene. As described below, this additional complexity in *S. cerevisiae* helped the analysis of TOR signaling because it allowed differentiating two functionally different signaling branches on the basis of different requirements for the two TORs.

It should be noted that there is no evidence to indicate that FKBP has a role in normal TOR signaling, *i.e.*, in the absence of rapamycin. Rapamycin hijacks or corrupts FKBP to interact with TOR. In addition, some have speculated that rapamycin mimics an endogenous metabolite that normally regulates TOR with or without FKBP. Although this would provide an explanation for the evolution of the mechanism of action of rapamycin, no evidence has been reported for an endogenous rapamycin-like compound or for such a mode of TOR regulation.

All TORs have a similar domain structure (Figure 1A). The domains found in TOR—in order from the N to the C terminus of TOR—compose the so-called HEAT repeats, the FAT domain, the FRB domain, the kinase domain, and the FATC domain (Schmelzle *et al.* 2002). The HEAT repeats (originally found in **h**untingtin, **e**longation factor 3, the **A** subunit of PP2A, and **T**OR1) consist of ~20 HEAT motifs,

each of which is ~40 residues that form a pair of interacting antiparallel α-helices (Andrade and Bork 1995; Perry and Kleckner 2003). The HEAT repeats occupy the N-terminal half of TOR and are the binding region for subunits of the TOR complexes (Wullschleger *et al.* 2005) (see below). The central FAT domain (~500 residues) and the extreme C-terminal FATC domain (~35 residues), flanking the FRB and kinase domains, are always paired and found in all PIKK family members (Alarcon *et al.* 1999; Bosotti *et al.* 2000; Dames *et al.* 2005). The FRB domain (~100 residues) is the FKBP–rapamycin-binding region. All rapamycin resistance-conferring *TOR* mutations fall within the FRB domain, thereby directly preventing the binding of FKBP–rapamycin without otherwise affecting TOR activity (Heitman *et al.* 1991a; Cafferkey *et al.* 1993; Helliwell *et al.* 1994; Stan *et al.* 1994; Chen *et al.* 1995; Lorenz and Heitman 1995; Choi *et al.* 1996). Interestingly, all the original rapamycin-resistance conferring mutations in *TOR1* and *TOR2* are missense mutations confined to a single, equivalent codon encoding a critical serine residue (Ser1972Arg or Ser1972Asn in TOR1 and Ser1975Ile in TOR2) (Cafferkey *et al.* 1993; Helliwell *et al.* 1994), which explains why the rapamycin-resistance *TOR* mutations were rare. Recreating an equivalent mutation (Ser2035Ile) in mammalian TOR (mTOR) was instrumental in demonstrating that mTOR is the target of FKBP–rapamycin in mammalian cells (Brown *et al.* 1995). Thus, the early rapamycin-resistant yeast mutants turned out to be very informative. They not only identified TOR, but also identified the FKBP–rapamycin-binding site in TOR and contributed to elucidating the mechanism of action of rapamycin. The kinase domain is the catalytic domain and resembles the kinase domain of PI3K and PI4K lipid kinases. Despite high interest in a structure of the kinase domain, no such structure exists for any TOR, which is likely due to technical difficulties in expressing this domain for structural studies. In the absence of a true model, a homology model based on the crystal structure of related PI3K has been elaborated (Sturgill and Hall 2009). A number of groups have identified activating, missense mutations in *S. cerevisiae* and *Schizosaccharomyces pombe* TORs (Reinke *et al.* 2006; Urano *et al.* 2007; Ohne *et al.* 2008). These mutations fall within the FAT, FRB, and kinase domains, and, interestingly, one of the hotspots in the kinase domain corresponds to a region for oncogenic mutations in PI3K (Sturgill and Hall 2009; Hardt *et al.* 2011).

In the mid-1990s, research in the TOR field focused on elucidating the cellular roles of TOR1 and TOR2. It was found that TOR1 and TOR2 play a central role in controlling cell growth as part of two separate signaling branches. Although structurally similar, TOR1 and TOR2 are not functionally identical (Kunz *et al.* 1993; Helliwell *et al.* 1994). Combined disruption of *TOR1* and *TOR2*, or rapamycin treatment, mimics nutrient deprivation including causing a G0 growth arrest within one generation (Barbet *et al.* 1996). Disruption of *TOR1* alone has little-to-no effect, and disruption of *TOR2* alone causes cells to arrest growth

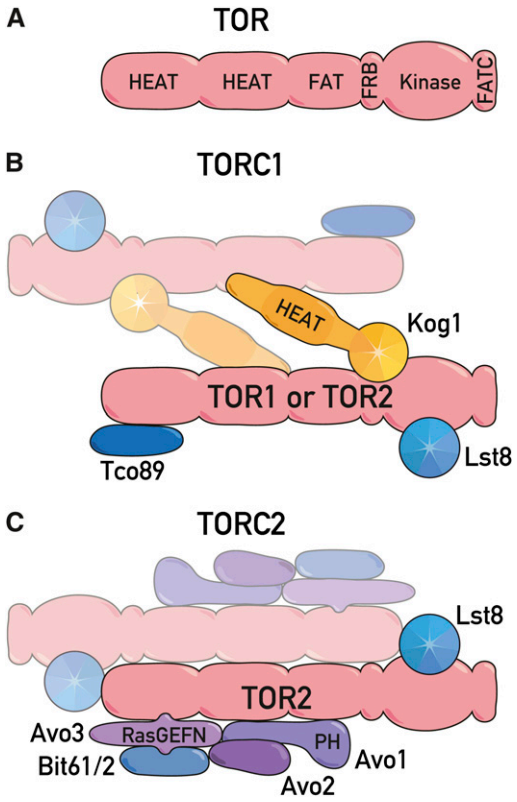

Figure 1 (A) Conserved domain structure of TOR. The N-terminal half of TOR is composed of two blocks of ~20 HEAT repeats, 40 aa that form pairs of interacting antiparallel α-helices. The ~500-aa FAT (FRAP-ATM-TRRAP) domain contains modified HEAT repeats. Missense mutations in the ~100-aa FRB (FKBP12-rapamycin-binding) domain confer complete resistance to rapamycin. The kinase domain phosphorylates Ser/Thr residues in protein substrates, but at the sequence level resembles the catalytic domain of phosphatidylinositol kinases. The ~35-aa FATC domain is always found C-terminal to the FAT domain and is essential for kinase activity. (B) Composition of TOR complex 1. TORC1 is ~2 MDa in size and contains Kog1, Tco89, Lst8, and either TOR1 or TOR2. The HEAT repeats found in Kog1 and the seven-bladed propellers of the WD-40 repeats found in Kog1 and Lst8 are depicted. The binding of Kog1 to TOR is complex, involving multiple domains on each protein. Lst8 binds to the kinase domain of TOR. Each component is likely present in two copies. (C) Composition of TOR complex 2. TORC2 is ~2 MDa in size and contains Avo1, Avo2, Avo3, Bit61, and/or its paralog Bit2, Lst8, and TOR2 but not TOR1. The RasGEFN domain of Avo3 and the PH domain of Avo1 are indicated. Each component is likely present in two copies.

within a few generations as small-budded cells in the G2/M phase of the cell cycle and with a randomized actin cytoskeleton (Kunz *et al.* 1993; Helliwell *et al.* 1994, 1998a; Schmidt *et al.* 1996). These and other findings led to the model that TOR2 has two essential functions: one function is redundant with TOR1 (TOR shared) and the other function is unique to TOR2 (TOR2 unique) (Hall 1996; Helliwell *et al.* 1998a). As described below, these two TOR2 functions turned out to be two separate signaling branches (each corresponding to a structurally and functionally distinct TOR complex) that control cell growth in different ways (Barbet *et al.* 1996; Schmidt *et al.* 1997, 1998; Bickle *et al.* 1998; Helliwell *et al.* 1998a; Loewith *et al.* 2002; Loewith and Hall

2004; De Virgilio and Loewith 2006; Breitkreutz *et al.* 2010; Kaizu *et al.* 2010).

The early characterization of *TOR* disruptions and rapamycin treatment led to two more important conclusions. First, as described in more detail below, the finding that TOR inhibition mimics starvation led to the notion that TOR controls cell growth in response to nutrients (Barbet *et al.* 1996; Rohde *et al.* 2001). Subsequent studies confirmed this notion and demonstrated that TOR in higher eukaryotes also controls cell growth in response to nutrients; *i.e.*, TOR is conserved in structure and function (Thomas and Hall 1997; Hara *et al.* 1998; Schmelzle and Hall 2000). Second, the observation that inhibition specifically of the TOR-shared signaling branch (disruption of both *TOR*s but not of *TOR2* alone) or rapamycin treatment mimics starvation suggested that only the TOR-shared pathway is nutrient responsive and rapamycin sensitive (Zheng *et al.* 1995; Barbet *et al.* 1996; Schmidt *et al.* 1996; Rohde *et al.* 2001). The molecular basis of these findings would remain a mystery until the discovery of the two structurally and functionally distinct TOR complexes (see below).

The realization that TOR controls growth (increase in cell size or mass) was a particularly important development (Barbet *et al.* 1996; Thomas and Hall 1997; Schmelzle *et al.* 2002). Rapamycin or loss of *TOR* function causes a cell cycle arrest, and TOR was thus originally thought to be a controller of cell division (increase in cell number). Furthermore, at that time, growth was largely thought to be controlled passively: *i.e.*, the simple availability of nutrients (building blocks) led to cell growth. As described below, the realization that TOR controls many cellular processes that collectively determine mass accumulation, combined with the fact that there was no direct role for TOR in the cell cycle machinery then being characterized, led to the notions that TOR controls growth and that growth is thus actively controlled. The originally confusing defect in cell cycle progression observed upon TOR inhibition is in fact an indirect effect of growth inhibition: a growth defect is dominant over cell cycle progression.

Since the late 1990s, many groups have been characterizing the two separate TOR2-signaling branches. It was found that the TOR-shared signaling branch is composed of various effector pathways that control a wide variety of readouts that collectively determine the mass of the cell. The readouts controlled by this branch include protein synthesis and degradation, mRNA synthesis and degradation, ribosome biogenesis, nutrient transport, and autophagy (Schmelzle and Hall 2000). This branch is viewed as mediating temporal control of cell growth. The TOR2-unique branch controls the polarized organization of the actin cytoskeleton, endocytosis, and sphingolipid synthesis. This second branch is viewed as mediating spatial control of cell growth, on the basis largely of early work showing that it controls the actin cytoskeleton. Thus, the logic of the two branches appears to be to integrate temporal and spatial control of cell growth (Loewith and Hall 2004). However, this way of thinking about the two branches has subsided in recent years as the TOR2-unique pathway was shown to control sphingolipid synthesis and endocytosis in addition to the actin cytoskeleton (Powers *et al.* 2010).

Another major breakthrough in the TOR field occurred in 2002: the identification of the two multiprotein complexes termed TOR complex 1 (TORC1) and TORC2 (Loewith *et al.* 2002; Wedaman *et al.* 2003; Reinke *et al.* 2004; Wullschleger *et al.* 2006). The two structurally and functionally distinct TOR complexes were biochemically purified from yeast cells and subsequently shown to correspond to the two genetically defined TOR-signaling branches. TORC1, which contains either TOR1 or TOR2 and is rapamycin sensitive, mediates the TOR-shared pathway. TORC2, which specifically contains TOR2 and is rapamycin insensitive, mediates the TOR2-unique pathway. The TORCs were a major breakthrough because they provided a molecular basis for the functional complexity and selective rapamycin sensitivity of TOR signaling. The biochemical identification of the TORCs and the genetic definition of the two signaling branches also, gratifyingly, cross-validated each other such that there is a high level of confidence in the current "two branch-two complex" model of TOR signaling. The subsequent identification of TORCs in other eukaryotes, including plants, worms, flies, and mammals (Table 1), showed that the two complexes, like TOR itself, are conserved and gave further support to the above model (Hara *et al.* 2002; Kim *et al.* 2002; Loewith *et al.* 2002; Jacinto *et al.* 2004; Sarbassov *et al.* 2004). Below we focus on the structure, function, and regulation of the two TOR complexes. We discuss some downstream readouts of the TORCs that were originally described before the discovery of the TORCs but are now retroactively attributed to TORC1 or TORC2 on the basis of their TOR requirement or rapamycin sensitivity.

TOR Complex 1

Composition of TOR complex 1

TORC1 consists of Kog1, Lst8, Tco89, and either TOR1 or TOR2 (Figure 1B) (Loewith *et al.* 2002; Wedaman *et al.* 2003; Reinke *et al.* 2004). Gel filtration chromatography (R. Loewith, W. Oppliger, and M. Hall, unpublished results) indicated that TORC1 has a size of ~2 MDa, suggesting that the entire complex is likely dimeric. This would be consistent with the dimeric structures proposed for TORC2 (Wullschleger *et al.* 2005) and mTORC1 (Yip *et al.* 2010). The names of mammalian and invertebrate orthologs of TORC1 subunits and the salient features of *S. cerevisiae* TORC1 subunits are summarized in Table 1 and Table 2, respectively. Although all subunits are thought to act positively with TOR1/2 *in vivo*, by and large their functions await characterization. In the presence of rapamycin, all components of TORC1 can be coprecipitated with FKBP12 (Loewith *et al.* 2002), demonstrating that, unlike mammalian TORC1 (Yip *et al.* 2010), the structural integrity

Table 1 TORC1, TORC2, and EGO complex orthologs in various genera

S. cerevisiae	*S. pombe*	*C. albicans*	*D. discoideum*	*A. thaliana*	*C. elegans*	*D. melanogaster*	Mammals
				TORC1			
TOR1 or TOR2	Tor1 or Tor2	Tor1	Tor	TOR	TOR/let-363	TOR	mTOR
Kog1/Las24	Mip1	Kog1	Raptor	RAPTOR1A and RAPTOR1B	daf-15	Raptor	Raptor
Lst8	Wat1/Pop3	Orf19.3862	lst8?	*AT2G22040* *AT3G18140*	lst-8?	*CG3004*	mLST8
Tco89	Tco89	Tco89	pcr25kl1p3887	—	—	—	—
—	Toc1	—	—	—	—	—	—
—	—	—	—	—	—	—	PRAS40
—	—	—	—	—	—	—	DEPTOR
				TORC2			
TOR2	Tor1 or Tor2	Tor1	tor	TOR	TOR/let-363	TOR	mTOR
Avo1	Sin1	orf19.5221	piaA		sinh-1	Sin1	mSIN1
Avo2	—	Avo2		—	—	—	—
Avo3/Tsc11	Ste20	Tsc11	rip3		rict-1	Rictor	Rictor
Lst8	Wat1/Pop3	Orf19.3862	lst8	*AT2G22040* *AT3G18140*	lst-8	*CG3004*	mLST8
Bit61	Bit61	—	—	—	—	—	PRR5/Protor
—	—	—	—	—	—	—	DEPTOR
				EGO complex			
Gtr1	Gtr1	Gtr1	ragA	—	raga-1	RagA	RagA,B
Gtr2	Gtr2	Gtr2	ragC	—	ragc-1	RagC	RagC,D
Ego1/Meh1/Gse2	—	—	—	—	—	*CG14184*	LAMTOR1/p18
Ego3/Slm4/Nir1/Gse1	—	—	—	—	lamtor-2, ?	*CG5189*, *CG5110*	LAMTOR2/p14, LAMTOR3/ MP1

Orthologs listed are from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans*, *Dictyostelium discoideum*, *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Dictyostelium melanogaster*, and mammals. P-POD: Princeton Protein Orthology Database/BLAST. We note that TORC2 appears to be absent in plants, *e.g., A. thaliana*. —, no demonstrated/obvious ortholog.

Table 2 Salient features of TORC1 components

Protein	Size	Motifs/domains	Potential function
TOR1	2470 aa	HEAT repeats, FAT domain, FRB domain, kinase domain, and FATC domain	Protein kinase, scaffold
TOR2	2474 aa	HEAT repeats, FAT domain, FRB domain, kinase domain, and FATC domain	Protein kinase, scaffold
Kog1	1557 aa	An N-terminal conserved region 4, HEAT repeats, 7 C-terminal WD-40 repeats	Present substrate to TOR
Tco89	799 aa	None obvious	Receive signals from EGO complex
Lst8	303 aa	7 WD-40 repeats	Stabilize kinase domain

of yeast TORC1 is not compromised by this macrolide. Despite recent molecular reconstructions from low resolution (25 Å) electron microscopy of a TOR1–Kog1 subcomplex (Adami *et al.* 2007), the molecular mechanism by which binding of FKBP-rapamycin inhibits TORC1 activity is enigmatic and remains a fascinating question.

Localization of TORC1

Tagging of Kog1, Tco89, Lst8, and TOR1 with GFP demonstrates that TORC1 is concentrated on the limiting membrane of the vacuole (Urban *et al.* 2007; Sturgill *et al.* 2008; Berchtold and Walther 2009; Binda *et al.* 2009). These observations are consistent with previous studies that localized TORC1 via immunogold electron microscopy and cellular fractionation (Chen and Kaiser 2003; Reinke *et al.* 2004). Artificial tethering of a TORC1 peptide substrate to the vacuole demonstrates that vacuole-localized TORC1 is catalytically competent (Urban *et al.* 2007). This localization appears to be constitutive (Binda *et al.* 2009), suggesting that changes in "geography" play no obvious role in regulating yeast TORC1-signaling output. The yeast vacuole is a major nutrient reservoir and TORC1 signaling is responsive to nutrient cues (see below). Thus, vacuolar localization of TORC1 seems logical. Although convincing, these studies do not exclude the possibility that a fraction of TORC1 may also be active elsewhere in the cell. Li *et al.* (2006), for example, have reported that TOR1 dynamically associates with the rDNA locus to regulate 35S rRNA transcription.

Upstream of TORC1

Physiological regulators (carbon, nitrogen, phosphate, stress, caffeine): A major breakthrough in the TOR field came with the observation that rapamycin treatment alters yeast physiology in much the same way as nutrient starvation (Barbet *et al.* 1996). Like starvation, exposure of yeast cells to rapamycin results in a dramatic drop in protein synthesis, induction of autophagy, and exit from the cell cycle and entrance into a quiescent G0 state. This was the first indication that TOR, actually TORC1, might regulate growth downstream of nutrient cues. This hypothesis was strengthened when TORC1, in response to nitrogen and carbon cues, was found to promote the sequestration of several nutrient-responsive transcription factors in the cytoplasm (Beck and Hall 1999). Consistently, transcriptome profiling demonstrated a highly similar transcriptional response of yeast cells exposed to rapamycin, nutrient starvation, or noxious stressors (Cardenas *et al.* 1999; Hardwick *et al.* 1999; Komeili *et al.* 2000; Shamji *et al.* 2000; Gasch and Werner-Washburne 2002). Although suggestive, these observations provided only correlative evidence that TORC1 activity is regulated in response to environmental cues. Characterization of a *bona fide* substrate of TORC1 allowed this model to be tested directly.

As detailed below, Sch9 presently remains the best-characterized substrate of TORC1, and monitoring its phosphorylation by Western blotting serves as a convenient proxy for TORC1 activity. In addition to exposure to rapamycin, Sch9 is rapidly dephosphorylated in cells experiencing acute starvation of carbon, nitrogen, phosphate, or amino acids (Urban *et al.* 2007; Binda *et al.* 2009). These and other observations confirm that TORC1 is responsive to both the abundance and the quality of nutrients in the environment; but, with few exceptions (see *The EGO complex*), how nutrient cues are sensed and how this information is transduced to TORC1 remain unknown.

TORC1 activity is also regulated in response to noxious stressors. When cells are subjected to various stress conditions, including high salt, redox stress, a shift to a higher temperature, or caffeine, Sch9 phosphorylation is reduced dramatically (Kuranda *et al.* 2006; Urban *et al.* 2007). With the exception of caffeine, which directly inhibits TORC1

Table 3 Salient features of EGO Complex components

Protein	Size	Motifs/domains	Potential function
Gtr1	310 aa	Ras-family GTPase	GTP-bound form activates TORC1
Gtr2	341 aa	Ras-family GTPase	GDP-bound form activates TORC1
Ego1/Meh1/ Gse2	184 aa	N-terminal palmitoylation/myristolation[a]	Vacuolar recruitment
Ego3/Slm4/Nir1/Gse1	162 aa	Transmembrane domain, PtdIns(3,5)P2 binding[a]	Vacuolar recruitment

[a] Described in Dubouloz *et al.* (2005), Hou *et al.* (2005), and references therein.

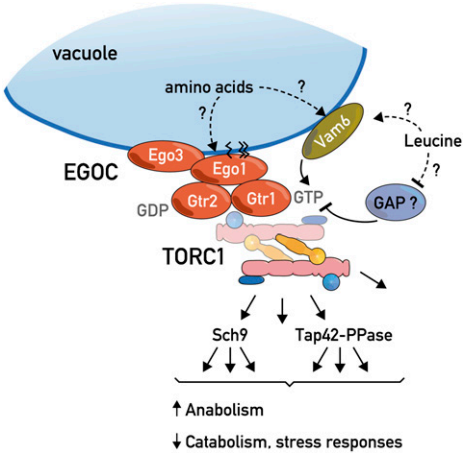

Figure 2 The EGO complex is a major regulator of TORC1. The EGO complex (EGOC) is composed of four proteins: the palmitoylated and myristolated protein Ego1, the transmembrane protein Ego3, and two Ras-family GTPases, Gtr1 and Gtr2. Like TORC1, the EGO complex is localized to the vacuolar membrane where it appears to sense/respond to intracellular leucine levels and potentially to intravacuolar amino acid levels. Vam6 has been identified as a guanine nucleotide exchange factor for Gtr1 but no other GEFs or GAPS for this GTPase system have been reported. In the $Gtr1^{GTP}$ and $Gtr2^{GDP}$ configuration, the EGO complex somehow activates TORC1; the reverse conformation inactivates TORC1. Activated TORC1, via its two main effector branches, the AGC kinase Sch9 and the Tap42-PP2a and PP2a-like protein phosphatases, stimulates growth by favoring anabolic processes and by antagonizing catabolic processes and stress-response programs.

kinase activity (Kuranda *et al.* 2006; Reinke *et al.* 2006; Wanke *et al.* 2008), how environmental stress signals are transduced to TORC1 is also unclear.

The EGO complex: When environmental conditions are inappropriate for growth, cells stop dividing, slow their metabolism, induce the expression of stress-responsive proteins, and accumulate energy stores. This nondividing but metabolically active state is known as quiescence (G0). How cells enter into quiescence is relatively well characterized. In contrast—and despite its medical relevance (inappropriate exit from quiescence can lead to cancer or reactivation of a latent infection)—how quiescent cells reinitiate growth is poorly understood. To shed light on this process, a clever screen was performed to identify mutants that are unable to **e**scape from rapamycin-induced **gro**wth arrest (*EGO*) mutants (Dubouloz *et al.* 2005). This and a follow-up study (Binda *et al.* 2009) identified the EGO complex as an important regulator of TORC1.

The EGO complex is composed of four proteins: Ego1, Ego3, Gtr1, and Gtr2 (Table 3 and Figure 2). Gtr1 and Gtr2 are Ras-family GTPases and orthologs of the metazoan Rag GTPases (Kim *et al.* 2008; Sancak *et al.* 2008) (Table 1). Although they lack obvious sequence homologies, Ego1 and Ego3 are likely the functional homologs of vertebrate p18 (LAMTOR1) and p14 + MP1 (LAMTOR2 + LAMTOR3), respectively, which function together as the "Ragulator" complex (Kogan *et al.* 2010; Sancak *et al.* 2010). Ragulator and the Rags mediate amino acid sufficiency signals to mTORC1 (reviewed in Kim and Guan 2011). Like its mammalian counterpart, the EGO complex resides on the vacuolar/lysosomal membrane and is thought to couple amino acid signals to TORC1 (Binda *et al.* 2009). Curiously, the $Gtr1^{GTP}$ $Gtr2^{GDP}$ combination activates TORC1 with the nucleotide-binding status of Gtr1 seemingly dominant over the nucleotide-binding status of Gtr2.

TORC1 activity in both metazoans and yeast appears to be particularly responsive to glutamine (Crespo *et al.* 2002) and the branched-chain amino acid leucine (Binda *et al.* 2009; Cohen and Hall 2009). In yeast, leucine starvation destabilizes Gtr1-TORC1 association and causes a reduction in Sch9 phosphorylation whereas GTP-locked $Gtr1^{Q65L}$ remains bound to TORC1 and Sch9 dephosphorylation is delayed in cells expressing this mutant (Binda *et al.* 2009). Loss of Gtr1 results in reduced Sch9 phosphorylation and slow growth whereas GDP-locked $Gtr1^{S20L}$ is dominant negative. When $Gtr1^{S20L}$ is expressed as the sole version of Gtr1, cells are extremely sick. This near inviability is suppressed by deletion of the *TCO89* gene encoding the TORC1 subunit Tco89. Collectively, these observations suggest that the EGO complex can both positively and negatively regulate TORC1 activity via Tco89. The fact that the EGO complex can negatively regulate TORC1 activity seems to be at odds with the current metazoan model according to which the EGO complex counterpart serves only to localize TORC1 to the vacuole. Indeed, in contrast to the results obtained in metazoans, in yeast, TORC1 appears to stably localize to the vacuolar membrane regardless of nutrient conditions. Thus, how the EGO complex influences TORC1 activity remains a mystery although the crystal structure of the Gtr1–Gtr2 complex, reported very recently, provides some mechanistic insights (Gong *et al.* 2011).

Also mysterious are the mechanisms by which amino acid sufficiency modulates Gtr1/2 guanine nucleotide loading. Given its localization, it is tempting to postulate that the EGO complex responds to levels of intravacuolar amino acids, possibly via the recently described Gtr1 guanine–nucleotide exchange factor (GEF) Vam6/Vps39 (Binda *et al.* 2009). It is equally plausible, however, that this signal is mediated by an as-yet-unidentified GTPase-activating protein (GAP) activity. Consistent with the conserved function of the EGO/Ragulator complex, and like its yeast ortholog, hVPS39 has been found to function positively upstream of mTORC1 (Flinn *et al.* 2010).

Feedback loop/ribosome biogenesis homeostasis: Although most recognized as a target of signals emanating from extracellular nutrients and noxious stresses, it is becoming increasingly apparent that TORC1 also responds to intracellular cues. In addition to the sensing of intracellular amino acids as described above, outputs from distal effectors also regulate TORC1 in apparent feedback loops. For example, in both yeast and mammalian cells, it is well documented that TORC1 activity stimulates translation initiation

(Wullschleger *et al.* 2006). Interestingly, inhibition of translation with cycloheximide causes a pronounced increase in (m)TORC1 activity presumably by triggering an increase in the concentration of free amino acids in the cytoplasm (Beugnet *et al.* 2003; Urban *et al.* 2007; Binda *et al.* 2009). Ribosome biogenesis (described in more detail below) is a second example. TORC1 regulates ribosome biogenesis in part via two substrates, Sch9 and the transcription factor Sfp1. Reduced ribosome biogenesis resulting from deletion of *SCH9* or *SFP1* results in a dramatic increase in TORC1 activity (Lempiainen *et al.* 2009). It is possible that blocking ribosome biogenesis, like translation inhibition, causes an increase in free amino acids that subsequently activates TORC1. Alternatively, other mechanisms could be at play. Regardless of mechanism, such feedback loops provide an elegant means by which growth homeostasis can be maintained by TORC1.

Downstream of TORC1

In general terms, when growth conditions permit, TORC1 is active and its signals promote the accumulation of cellular mass. However, as both proximal and distal TORC1 effectors continue to be described, the extent of this temporal regulation of growth control is only starting to be appreciated.

Proximal TORC1 effectors: *Characterization of Sch9 as a TORC1 substrate:* Arguably, the best-characterized substrates of both yeast and metazoan TOR complexes are the AGC family kinases. This rather well-studied family of kinases is so named on the basis of its mammalian members PKA, PKG, and PKC (Pearce *et al.* 2010). Typically, activation of AGC family kinases requires phosphorylation of two conserved regulatory motifs, the "T," or "activation," loop located in the catalytic domain and the "hydrophobic" motif found toward the C terminus. Phosphorylation of these motifs helps stabilize the kinase domain in an active conformation. Several AGC family kinases additionally contain a "turn" motif located between the kinase domain and the hydrophobic motif, phosphorylation of which is thought to promote protein stability. While the T loop is phosphorylated by PDK1 or its ortholog Pkh in mammalian or yeast cells, respectively, phosphorylation of the hydrophobic and possibly the turn motifs is often mediated by TORC1 or TORC2.

Analogous to S6K for mTORC1, the AGC kinase Sch9 was recently found to be a direct substrate for yeast TORC1 (Powers 2007). Six target sites in the C terminus of Sch9 are phosphorylated by TORC1: Thr737 found in a classical hydrophobic motif; Thr723 and Ser726, Ser/Thr-Pro sites found in what appears to be a turn motif; Ser758 and Ser765 found in sequences that resemble the hydrophobic motif; and Ser711 in a region that partially resembles a hydrophobic motif. TORC1-mediated phosphorylation is necessary for Sch9 activity. Replacing the target amino acids with alanine yields a nonfunctional Sch9, whereas replacing them with a phosphomimetic residue confers constitutive kinase activity, *i.e.*, activity even in the absence of TORC1 (Urban *et al.* 2007). Presumably, phosphorylation of the turn motif helps to stabilize Sch9 while phosphorylation of the hydrophobic motif stabilizes Sch9 in an active conformation. Curiously, although their *in vivo* functions are unknown, *in vitro* TORC1 preferentially phosphorylates Ser758 and Ser765 within the hydrophobic-like motifs (R. Loewith, unpublished results). That TORC1 can phosphorylate amino acids found within such diverse sequence contexts, which is rather atypical for protein kinases, is also curious.

Characterization of Tap42-PP2A as a TORC1 effector: In addition to Sch9, TORC1 also regulates type 2A (Pph21, Pph22, and Pph3—generically PP2Ac) and 2A-related phosphatases (Sit4, Ppg1). These partially redundant yet pleiotropic enzymes are notoriously difficult to study. Analysis of Sit4 function, and therefore of TORC1 function, is further complicated by strain-dependent polymorphisms at the *SSD1* (**S**uppressor of ***SIT4*** **D**eletion) locus (Reinke *et al.* 2004).

A role for these phosphatases downstream of TORC1 was first described by the Arndt lab (Di Como and Arndt 1996). In this work, a subpopulation of these enzymes was found to interact in a TORC1-dependent manner with a regulatory protein known as Tap42. Rrd1 and Rrd2, two peptidyl-prolyl *cis/trans* isomerases, were subsequently also found to be present in these Tap42 complexes (Zheng and Jiang 2005; Jordens *et al.* 2006). Work, done in large part by the Jiang group, posits that when TORC1 is active, Tap42 is phosphorylated and bound tightly to the phosphatase–Rrd complex (Di Como and Arndt 1996; Jiang and Broach 1999; Zheng and Jiang 2005). Inactivation of TORC1 results in Tap42 dephosphorylation and a weakened association with phosphatases that presumably results in their activation and/or change in substrate preference (Duvel *et al.* 2003; Yan *et al.* 2006). How TORC1 maintains Tap42 phosphorylation is mechanistically unclear. It may phosphorylate Tap42 directly (Jiang and Broach 1999), or it may act via the **T**ap42 **i**nteracting **p**hosphoprotein Tip41 (Jacinto *et al.* 2001). Interestingly, Tip41 has been proposed to both antagonize and cooperate with Tap42 in controlling TORC1 signaling (Jacinto *et al.* 2001; Kuepfer *et al.* 2007).

Although the mechanisms coupling TORC1 to Tap42–PPase complexes remain to be elucidated, genetic arguments clearly position Tap42 as a prominent effector of TORC1. Specifically, several alleles of *TAP42* (*e.g.*, *TAP42-11*) that confer strong resistance to rapamycin by blocking a subset of rapamycin-induced readouts have been identified (Di Como and Arndt 1996; Duvel *et al.* 2003).

Curiously, *TAP42-11* does not provide rapamycin resistance in all strain backgrounds. However, co-expression of genetically activated Sch9 (described above) in rapamycin-sensitive *TAP42-11* backgrounds results in a very strong synthetic resistance to rapamycin (Urban *et al.* 2007). From this observation, it appears that Sch9 and Tap42-PPase complexes are major effector branches downstream of TORC1 with each branch, at least in some backgrounds, performing

one or more essential function. The readouts mediated by these two TORC1 branches are discussed below.

Other TORC1 substrates: In addition to the regulation of these two major effector branches, TORC1 has been reported to directly phosphorylate other substrates including Sfp1(Lempiainen *et al.* 2009), Gln3 (Bertram *et al.* 2000), and Atg13 (Kamada *et al.* 2010). The roles that these proteins play downstream of TORC1 are discussed below.

Tyers and colleagues have recently defined a global protein kinase and phosphatase interaction network in yeast (Breitkreutz *et al.* 2010). This study, consisting of affinity purification followed by mass spectrometry, included TOR1 and TOR2. They found and confirmed that TORC1 physically interacts with the following proteins: Mks1, a protein involved in retrograde (RTG) mitochondria-to-nucleus signaling (see below); curiously, FMP48, an uncharacterized protein presumed to localize to the mitochondria (Reinders *et al.* 2006); Npr1, a protein kinase involved in the intracellular sorting of nutrient permeases (see below); Ksp1, a protein kinase involved in nutrient-regulated haploid filamentous growth (Bharucha *et al.* 2008); Nap1, a chromatin assembly factor and a mitotic factor involved in regulation of bud formation (Calvert *et al.* 2008); Nnk1, the **n**itrogen **n**etwork **k**inase presumably involved in intermediate nitrogen metabolism (Breitkreutz *et al.* 2010); Sky1, an Ser/Arg domain kinase involved in pre-mRNA splicing (Shen and Green 2006); and Bck1 and Kdx1, which are involved in MAPK signaling (Breitkreutz *et al.* 2010). Given their physical interaction with TORC1, all of these proteins, in addition to multiple other, as-yet-unconfirmed interactors, are potential substrates (or regulators) of TORC1. These results underscore the central role that TORC1 plays in cell growth.

Distal readouts downstream of TORC1: *TORC1 promotes cell growth:* When environmental conditions are favorable, TORC1 coordinates the production and accumulation of cellular mass, *i.e.*, growth, via regulation of several processes.

Protein synthesis: The first realization that TORC1 serves to couple environmental cues to the cell growth machinery came with the observation that rapamycin treatment elicits a marked drop in protein synthesis by blocking translation initiation (Barbet *et al.* 1996). A major target for this regulation appears to be the translation initiation factor eIF2. Upon amino acid starvation or rapamycin treatment, the α-subunit of eIF2 is phosphorylated and this dominantly interferes with 5′CAP-dependent mRNA translation (reviewed in Hinnebusch 2005). TORC1 signals to eIF2α via both the Sch9 and Tap42-PPase branches. The sole eIF2α kinase is the conserved Gcn2 protein. Gcn2 binds and is activated by uncharged tRNAs that accumulate when cells are starved for an amino acid (detailed below). Gcn2 activity is also regulated by phosphorylation. Gcn2 phosphorylation on Ser577 reduces tRNA binding and, consequently, kinase activity. Treating cells with rapamycin elicits a rapid, Tap42-PPase-dependent dephosphorylation of Ser577 and, consequently, an increase in Gcn2 activity and a reduction in 5′CAP-dependent translation (Cherkasova and Hinnebusch 2003). It is possible that one or more Tap42-associated phosphatases directly dephosphorylates Ser577, but this has not been formally demonstrated. The nature of the kinase that phosphorylates Gcn2 Ser577 is unknown other than it is not Sch9 (M. Stahl and R. Loewith, unpublished results). Sch9 inhibition, however, also leads to eIF2α phosphorylation via an undefined pathway (Urban *et al.* 2007).

Studies with rapamycin suggest that, in addition to eIF2α, TORC1 may target additional translation factors such as the 5′CAP-binding protein (eIF4E) interacting proteins Eap1 and/or the eIF4G scaffold (Berset *et al.* 1998; Cosentino *et al.* 2000). Finally, recent phosphoproteomics studies (Huber *et al.* 2009; Loewith 2010; Soulard *et al.* 2010) have identified several translation-related proteins whose phosphorylation is altered by rapamycin treatment, suggesting that these factors could also couple TORC1 to protein synthesis.

Ribosome biogenesis: In optimal conditions, yeast cells grow and divide approximately every 100 min. Such rapid growth requires robust protein synthesis, which of course requires ribosomes. Indeed, rapidly growing yeast cells contain ∼200,000 ribosomes, implying that each cell must produce and assemble ∼2000 ribosomes per minute (Warner 1999). This is not a trivial feat as each ribosome contains 78 unique proteins (encoded by 137 *RP* genes) in addition to four rRNA molecules, three derived from the RNA Pol I-transcribed 35S pre-rRNA and one transcribed by RNA Pol III. Fifty percent of RNA Pol II transcription is devoted to ribosomal proteins. In addition, numerous protein and small RNA *trans*-acting factors, known as **ri**bosome **bi**ogenesis (RiBi) factors, are required for the correct processing, folding, assembly, nuclear export of pre-ribosomal particles to the cytoplasm, and final maturation events into 40S and 60S particles. The production of all these abundant molecules represents a huge energetic investment. Not surprisingly, yeast cells have developed elaborate measures to coordinate the expression of rRNA, tRNA, RPs, and RiBi factors in response to environmental conditions. Much of this regulation is mediated by TORC1 at the level of transcription. As ribosome biogenesis has clear links to diseases such as cancer, anemia, and aging, dissection of its regulation will undoubtedly have clinical ramifications (Lempiainen and Shore 2009).

In *S. cerevisiae*, the rDNA locus consists of ∼150 tandemly repeated transcription units on chromosome XII, and yet rRNA production is still limiting for cell growth (Warner 1999). Each of these rDNA units comprises the RNA polymerase III transcribed 5S rRNA gene, the intergenic spacer region, and the RNA Pol I-transcribed 35S rRNA gene, encoding the 35S precursor of the mature 18S, 5.8S, and 25S rRNAs. RNA Pol III also transcribes tRNA genes as well as several additional genes encoding small noncoding RNAs. In the late 1990s, it was reported that rapamycin results in

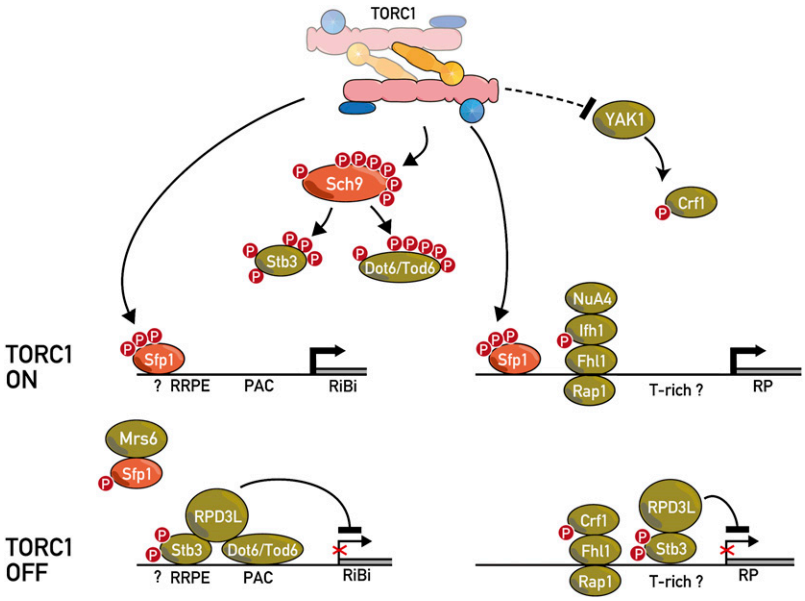

Figure 3 Control of RiBi and RP gene transcription by TORC1. RiBi factors are required for the proper expression, processing, assembly, export, and maturation of rRNA and RPs into ribosomes. This energetically costly procedure is under tight regulation, particularly at the transcription level. TORC1 regulates RiBi and RP gene transcription via multiple pathways: (1) TORC1 directly phosphorylates the Split Zn-finger transcription factor Sfp1, which presumably regulates its nuclear localization and/or binding to RP and possibly RiBi gene promoters to stimulate their expression. (2) Fhl1 and Rap1 bind constitutively to *RP* promoters. When TORC1 is active, phosphorylated Ifh1 binds to Fhl1 to stimulate transcription, possibly by recruiting the NuA4 histone acetyltransferase. When TORC1 is inactive, Yak1 phosphorylates Crf1, which subsequently outcompetes Ifh1 for binding to Fhl1. (3) Sch9 phosphorylates and thus inhibits Stb3 and the paralogs Dot6 and Tod6. Inhibition of TORC1/Sch9 results in the dephosphorylation of these three transcription repressors, which subsequently bind to *RRPE* and *PAC* elements found in RiBi promoters. Stb3 additionally binds RP promoters. Bound to promoters, these repressors recruit the RPD3L histone deacetylase complex to repress transcription.

a rapid and pronounced drop in 5S, 35S, and tRNA production (Zaragoza *et al.* 1998; Powers and Walter 1999). Recently, the relevant signaling pathways in this regulation have become clearer.

TORC1 regulates the accumulation of RNA Pol I transcripts at multiple levels. Processing of the 35S pre-rRNA occurs cotranscriptionally and is dependent on the presence of ribosomal proteins (Tschochner and Hurt 2003). The fast drop in RNA Pol I-dependent transcripts observed upon rapamycin treatment is apparently due to decreased translation (described above) of ribosomal proteins (Reiter *et al.* 2011). The majority of mRNAs being translated in a rapidly growing cell encode ribosomal proteins (Warner 1999), and thus a drop in translation will rapidly reduce the levels of free ribosomal proteins that are themselves needed stoichiometrically for processing of rRNA into pre-ribosome particles. rRNA that is not efficiently processed is immediately degraded, presumably to prevent imbalances in structural components of the ribosome. At later time points following rapamycin treatment, RNA Pol I no longer associates with the rDNA and transcription stops. This late effect could be the result of rapamycin-induced degradation of the essential RNA Pol I transcription factor Rrn3 (Claypool *et al.* 2004; Laferte *et al.* 2006; Reiter *et al.* 2011).

TORC1 regulates RNA Pol III apparently exclusively via Sch9 and a repressor protein named Maf1 (Upadhya *et al.* 2002; Oficjalska-Pham *et al.* 2006; Reina *et al.* 2006; Huber *et al.* 2009; Lee *et al.* 2009). Sch9 directly phosphorylates seven sites in Maf1 that prevent it from interacting with and thus inhibiting RNA Pol III (Vannini *et al.* 2010). Phosphomimetic variants of Maf1 clearly fail to associate with RNA Pol III, but, curiously, Sch9 inhibition still causes a reduction in RNA Pol III activity in these strains but not in *maf1Δ* strains. This and other observations suggest that an additional Sch9 target exists that, when dephosphorylated, represses RNA Pol III in a Maf1-dependent fashion (Huber *et al.* 2009; Michels 2011). Maf1 is conserved and also functions downstream of mTORC1 to regulate RNA Pol III activity. However, in mammalian cells, and perhaps in yeast cells too, Maf1 is directly phosphorylated by mTORC1 rather than by the Sch9 ortholog S6K1 (Wei *et al.* 2009; Wei and Zheng 2010; Michels 2011).

A total of 137 genes encode the 78 proteins that make up a yeast ribosome (most RPs are encoded by two genes yielding nearly identical proteins). TORC1 coordinately regulates the expression of these genes through several mechanisms (Figure 3) (Lempiainen and Shore 2009). A central component of this regulation is the Fhl1 protein (Lee *et al.* 2002; Martin *et al.* 2004; Schawalder *et al.* 2004; Wade *et al.* 2004; Rudra *et al.* 2005). Fhl1 has a forkhead DNA-binding domain, and its constitutive association to ribosomal protein gene (*RP*) promoters is facilitated by the DNA-binding protein Rap1 and the high mobility group protein Hmo1 (Hall *et al.* 2006; Berger *et al.* 2007). TORC1 regulates *RP* transcription by determining the association between Fhl1 and either one of two FHB-containing proteins, Ifh1 and Crf1. Both Ifh1 and Crf1 are phosphoproteins. When cells are growing and TORC1 is active, Ifh1 is phosphorylated and binds to Fhl1 to stimulate *RP* transcription. Conversely, inhibition of TORC1 results in the phosphorylation of Crf1, which displaces Ifh1 to repress *RP* transcription. The signaling events upstream of Ifh1 are not known, whereas TORC1 seems to signal to Crf1 via the Ras/PKA pathway target Yak1 (Martin *et al.* 2004). However, it should be noted that the crosstalk between TORC1 signals and Ras/PKA signals has been debated. While it is clear that hyperactivation of Ras/PKA can suppress many rapamycin-induced phenotypes (Schmelzle *et al.* 2004), suggesting that PKA is downstream of TORC1, it has also been proposed that TORC1 and PKA signal in parallel

pathways that impinge on common targets (Zurita-Martinez and Cardenas 2005; Ramachandran and Herman 2011). Recently, Soulard *et al.* (2010) have provided some clarification of this dilemma by proposing that TORC1 functions upstream of PKA but only for a subset of PKA targets. Thus, TORC1 may be both upstream and parallel to PKA.

TORC1-dependent regulation of *RP* gene transcription still occurs in the absence of the Fhl1/Ifh1/Crf1 system, suggesting the existence of additional regulatory mechanisms. One of these is the split zinc (Zn)-finger protein Sfp1 (Fingerman *et al.* 2003; Jorgensen *et al.* 2004; Marion *et al.* 2004; Lempiainen *et al.* 2009; Singh and Tyers 2009). TORC1 binds and directly phosphorylates Sfp1 to promote its binding to a subset of *RP* gene promoters. Curiously, unlike Sch9, TORC1-mediated Sfp1 phosphorylation appears to be insensitive to osmotic or nutritional stress, suggesting that TORC1 regulates these two substrates via very different mechanisms (Lempiainen *et al.* 2009). Sfp1 also interacts with the conserved Rab escort protein Mrs6, an essential protein functioning in membrane sorting (Lempiainen *et al.* 2009; Singh and Tyers 2009). Sfp1-Mrs6 association is important for the nuclear localization of Sfp1, but its functional implications are otherwise unclear. Intriguingly, this association may underlie the presently unexplained genetic and biochemical interactions between TORC1 and vesicular transport machineries (Aronova *et al.* 2007; Zurita-Martinez *et al.* 2007). Although physical interaction with *RiBi* promoters has not been reported, overexpression of Sfp1 causes a rapid upregulation of most *RiBi* genes, suggesting that Sfp1 also regulates this important regulon (Jorgensen *et al.* 2004). Better understood is the regulation of *RiBi* gene expression downstream of Sch9. *RiBi* promoters typically possess polymerase A and C (*PAC*) and/or **r**ibosomal **R**NA **p**rocessing **e**lement (*RRPE*) elements. *PAC* elements are bound by the myb-family transcription factors Dot6 and Tod6 (Freckleton *et al.* 2009; Zhu *et al.* 2009) whereas *RRPE* elements are bound by Stb3 (Liko *et al.* 2007). Stb3 seems to bind to T-rich elements in *RP* promoters as well (Huber *et al.* 2011). All three transcription factors are phosphorylated by Sch9 and thus are under TORC1 control (Lippman and Broach 2009; Liko *et al.* 2010; Huber *et al.* 2011). When TORC1 is inactivated, Dot6, Tod6, and Stb3 are dephosphorylated, allowing them to bind to their cognate promoter elements and recruit the RPD3L histone acetyltransferase complex to repress transcription.

In summary, TORC1 plays a central role in regulating ribosome biogenesis, particularly at the transcriptional level. However, it is now clear that TORC1 also influences ribosome biogenesis post-transcriptionally. Phosphoproteomics as well as more directed studies suggest that TORC1 regulates various catalytic steps of ribosome assembly *per se* (Honma *et al.* 2006; Huber *et al.* 2009; Loewith 2010). Phosphoproteomics and biochemical studies (Albig and Decker 2001; Grigull *et al.* 2004; Huber *et al.* 2009; Breitkreutz *et al.* 2010; Loewith 2010; Soulard *et al.* 2010) also suggest that TORC1 plays an active role in mRNA stability and, via its potential substrate Sky1, in pre-mRNA splicing. This observation is significant when one considers that 90% of all mRNA splicing events occur on *RP* transcripts (Warner 1999). Thus, TORC1 is well positioned to coordinate multiple aspects of ribosome biogenesis in response to growth stimuli. As introduced above, TORC1 activity is dramatically increased in *sfp1* and *sch9* cells (Lempiainen *et al.* 2009), suggesting that some aspect of ribosome biogenesis must also signal in a feedback loop to TORC1. It will be interesting to see what steps of ribosome biogenesis contribute to TORC1 regulation.

Regulation of cell cycle/cell size: Although distinct processes, cell growth and cell division are often intimately linked. Yeast cells, for example, commit to a new round of cell division only after attaining a critical size. This cell-size threshold is dictated in large part by environmental growth conditions (Cook and Tyers 2007). How cells couple environmental cues to the cell cycle machinery is fascinating but poorly understood. Interestingly, *sfp1* and *sch9* were the top two hits in a systematic search for mutations conferring small cell size (Jorgensen *et al.* 2002, 2004). This and follow-up observations demonstrated that ribosome biogenesis plays a major role in cell-size determination. These results further predict that environmental cues regulate the cell-size threshold via TORC1, *i.e.*, that poor growth conditions reduce the activity of TORC1 and subsequently the activities of Sfp1 and Sch9. Consequently, this would decrease ribosome biogenesis, which, in mysterious ways, would lower the cell-size threshold required for cell division. In contrast, acute inhibition of TORC1 with high concentrations of rapamycin leads to an arrest in G1 due to reduced translation of the cyclin Cln3 (Barbet *et al.* 1996) and a paradoxical increase in cell size. This increase in cell size is actually due to swelling of the vacuole as a consequence of increased autophagy (see below; *sfp1* or *sch9* deletions presumably do not induce autophagy).

Although best appreciated for its role in G1 regulation, TORC1 additionally regulates the transition through other phases of the cell cycle. TORC1 promotes S phase by maintaining deoxynucleoside triphosphate pools. Deoxynucleoside triphosphates are the obligate building blocks for DNA synthesis, and a role for TORC1 in their synthesis becomes apparent under conditions of DNA replication stress or DNA damage when elevated deoxynucleoside triphosphate pools are necessary for error-prone translesion DNA polymerases (Shen *et al.* 2007). Via the Tap42-PPase branch, TORC1 also influences the G2/M transition (Nakashima *et al.* 2008). Specifically, TORC1 regulates the subcellular localization of the polo-like kinase Cdc5. Cdc5 activity destabilizes Swe1, a kinase that phosphorylates and thus inactivates the mitotic cyclin-dependent kinase Cdc28. Inhibition of TORC1 mislocalizes Cdc5, causing an inappropriate stabilization of Swe1 and, consequently, inactivation of Cdc28 and prolonged G2/M. Although TORC1 signals likely impinge upon additional nodes in the cell division cycle (Huber *et al.* 2009; Soulard *et al.* 2010), the above

observations already exemplify the intricate connections between cell growth signals and the cell division cycle. Reciprocal, but less well described, cues and/or outputs from the cell division cycle regulate cell growth, likely in part via TORC1 (Goranov and Amon 2010).

TORC1 inhibits stress responses: In addition to stimulating anabolic processes, TORC1 also promotes growth by suppressing a variety of stress-response programs. Although essential for surviving environmental insults, activation of stress-responsive pathways is incompatible with rapid growth, and constitutive activation of these pathways generally results in cell death. As described below, the best-characterized stress-response programs under the influence of TORC1 are transcriptional in nature. However, it is clear that TORC1 also regulates post-transcriptional aspects of stress responses such as mRNA stability, protein trafficking, and the activities of metabolic enzymes.

Environmental stress response: Exposure of yeast cells to noxious stressors, including nutrient limitation and entry into stationary phase, elicits a stereotypic transcriptional response known as the environmental stress response (ESR) (Gasch and Werner-Washburne 2002). This includes ~300 upregulated genes that encode activities such as protein chaperones and oxygen radical scavengers that help cells endure stressful environments. The central components of this pathway are the Zn-finger transcription factors Msn2/4 and Gis1, the LATS family kinase Rim15, and the α-endosulfine family paralogs Igo1 and Igo2 (De Virgilio 2011). TORC1 via Sch9, and possibly also Tap42-PPase, promotes cytoplasmic anchoring of Rim15 to 14-3-3 proteins by maintaining Rim15 phosphorylated on Ser1061 and Thr1075 (Wanke *et al.* 2005, 2008). Inhibition of TORC1 results in nuclear localization of Rim15, which subsequently triggers the activation, in a poorly understood fashion, of the expression of Msn2/4- and Gis1-dependent ESR genes. However, TORC1 inhibition results in a marked turnover of mRNAs (Albig and Decker 2001), and, as noted above, in a dramatic drop in translation. Thus it would appear that increasing transcription of protein-coding genes in TORC1-inhibited cells would be futile as these mRNA would likely be degraded before ever being translated. This appears not to be the case as Rim15 phosphorylates Igo1 and its paralog Igo2, allowing them to associate with newly transcribed Msn2/4- and Gis1-regulated mRNAs to protect these transcripts from degradation via the 5′-3′ mRNA decay pathway (Talarek *et al.* 2010; Luo *et al.* 2011).

Nutrient uptake and intermediary metabolism: To best compete with other microbes in their environment, yeast have optimized the use of available nutrients to accommodate fast growth (De Virgilio and Loewith 2006). Although a wide variety of compounds can be utilized as carbon or nitrogen sources, yeast cells will exclusively assimilate preferred nutrient sources before using nonpreferred, suboptimal ones. To attain this dietary specificity, and to respond to nutritional stress, yeast cells carefully regulate the expression and sorting of their many (>270) membrane transporters, enabling them to selectively import only the desired nutrients (Van Belle and Andre 2001). In general terms, in good growth conditions, many high-affinity, substrate-selective permeases are expressed and sorted to the plasma membrane to actively pump in nutrients that are used directly in ATP production and/or anabolism of nitrogenous compounds. Shift to poor growth conditions results in the replacement of high-affinity permeases, which are targeted to the vacuole for degradation with few low-affinity, broad-specificity permeases that facilitate uptake of a wide range of carbon and nitrogenous compounds that can be catabolized by the cell. For example, in response to nitrogen starvation, the high-affinity tryptophan-specific permease, Tat2, localized to the plasma membrane, is ubiquitinated, endocytosed, and ultimately degraded. In contrast, the general amino acid permease Gap1 is re-routed to the plasma membrane instead of to the vacuole/endosomes. Although details are still emerging, TORC1 appears to regulate such permease-sorting events primarily via Tap42-PPase and its (potentially direct) effector Npr1 (Schmidt *et al.* 1998; Beck *et al.* 1999; De Craene *et al.* 2001; Jacinto *et al.* 2001; Soetens *et al.* 2001; Breitkreutz *et al.* 2010). Npr1 is a heavily phosphorylated, seemingly fungal-specific, Ser/Thr kinase that upon TORC1 inactivation is rapidly dephosphorylated and activated (Gander *et al.* 2008). Although genetic studies clearly imply a role for Npr1 in protein-sorting events, the mechanisms of this regulation have remained elusive. It is possible that the permeases themselves are Npr1 substrates. Indeed, several nutrient and cation permeases have been identified as rapamycin-sensitive phosphoproteins (Huber *et al.* 2009; Soulard *et al.* 2010). Also identified in these phosphoproteomics studies were several α-arrestin-related proteins. These phosphoproteins function as clathrin adaptor molecules and have been implicated in mediating the sorting fates of a number of different permeases; and, one, Aly2, has recently been reported to be an Npr1 substrate (Lin *et al.* 2008; Nikko *et al.* 2008; Nikko and Pelham 2009; O'Donnell *et al.* 2010). Whether this observation is indicative of a more general trend in Npr1-meditated permease trafficking remains to be seen.

TORC1 regulates permease activity by regulating not only permease localization but also expression. This was shown in early transcriptomics experiments, which clearly demonstrated that TORC1 regulates the expression of a large number of permeases and other factors required for the assimilation of alternative nitrogenous sources (Cardenas *et al.* 1999; Hardwick *et al.* 1999; Komeili *et al.* 2000; Shamji *et al.* 2000). TORC1 regulates the expression of **n**itrogen **c**atabolite **r**epression (NCR)-sensitive genes via the Tap42-PPase branch. The proteins encoded by these genes (*e.g.*, Gap1) enable cells to import and metabolize poor nitrogen sources such as proline and allantoin. In the presence of preferred nitrogen sources such as glutamine, glutamate, or ammonia, active TORC1 promotes the association of the GATA-family transcription factor Gln3 with its cytoplasmic anchor Ure2. Mechanistically, this involves both TORC1-dependent

and TORC1-independent regulation of Gln3, and possibly of Ure2, phosphorylation (Beck and Hall 1999; Cardenas *et al.* 1999; Hardwick *et al.* 1999; Carvalho and Zheng 2003; Georis *et al.* 2009a; Tate *et al.* 2009, 2010). Two other less-characterized GATA factors, Gat1 and Dal81, also have roles in the regulation of NCR-sensitive genes (Georis *et al.* 2009b).

In addition to the NCR pathway, TORC1 also regulates the expression of amino acid permeases by modulating the activity of the SPS-sensing pathway. This pathway consists of a plasma-membrane-localized sensor of external amino acids, **S**sy1, and two downstream factors, **P**tr3 and **S**sy5 (Ljungdahl 2009). Upon activation of the pathway, Ssy5 catalyzes an endoproteolytic processing event that cleaves and releases an N-terminal regulatory domain from two transcription factors, Stp1 and Stp2, the shortened forms of which translocate to the nucleus and activate the transcription of a number of amino acid permease-encoding genes. TORC1 via Tap42-PPase modulates this pathway by promoting the stability of Stp1 and thus the ability of cells to utilize external amino acids (Shin *et al.* 2009).

In contrast to the SPS-sensing pathway that is activated by amino acids, the Gcn4 transcription factor is activated upon amino acid starvation (Hinnebusch 2005). As mentioned above, rapamycin treatment or amino acid starvation results in a rapid decline in translation initiation by triggering phosphorylation of the α-subunit of eIF2. Although eIF2α phosphorylation results in the repression of bulk translation, due to the presence of four short upstream open reading frames in its leader sequence, the mRNA encoding Gcn4 is, in contrast, preferentially translated. Subsequent accumulation of Gcn4 protein leads to the transcriptional induction of nearly all genes encoding amino acid biosynthetic enzymes.

TORC1 also regulates amino acid biosynthesis, in particular glutamine and glutamate homeostasis, via the retrograde response pathway (Komeili *et al.* 2000; Crespo and Hall 2002; Crespo *et al.* 2002; Liu and Butow 2006). This signaling pathway serves to communicate mitochondrial dysfunction to the nucleus to induce an appropriate transcriptional response. In addition to hosting the aerobic energy production machinery, mitochondria are also the sites of amino acid precursor, nucleotide, and lipid production. Signals, possibly changes in glutamate or glutamine levels, emanating from dysfunctional mitochondria impinge upon a cytosolic regulatory protein, Rtg2. Thus activated, Rtg2 antagonizes the ability of Mks1 to sequester the heterodimeric bZip/HLH transcription factor complex composed of Rtg1 and Rtg3 in the cytoplasm. Allowed to enter the nucleus, Rtg1/3 activates genes encoding enzymes required for anaplerotic reactions that resupply tri-carboxylic acid cycle intermediates that have been extracted for biosynthetic reactions. Key among these intermediates is α-ketoglutarate, the precursor of glutamate and glutamine from which all nitrogen-containing metabolites evolve (Magasanik and Kaiser 2002). Both transcriptome-profiling experiments as well as genetic studies have implicated TORC1 as a negative regulator of Rtg1/3-dependent transcription (Komeili *et al.* 2000; Shamji *et al.* 2000; Chen and Kaiser 2003). However, it is presently unclear how TORC1 influences this pathway; TORC1 inhibition could indirectly influence retrograde response signaling via alterations in metabolite levels. Alternatively, the direct association between TORC1 and Mks1 observed by the Tyers group and described above and the fact that Mks1 is a rapamycin-sensitive phosphoprotein instead suggest that TORC1 could play a much more direct role in regulating this pathway (Liu *et al.* 2003; Breitkreutz *et al.* 2010). Finally, phosphoproteomics studies suggest that TORC1 regulates intermediate metabolism by directly altering the activities of metabolic enzymes, particularly those involved in the early steps of glycolysis (Loewith 2011).

Autophagy: As described above, starved cells express a suite of stress-responsive proteins to help them negotiate hostile environmental conditions. This new synthesis requires both energy and amino acids that yeast cells obtain by inducing autophagy. Autophagy refers to a variety of mechanisms by which cytosplasmic material, including proteins and lipids, is translocated to the vacuole and catabolized. Amino acids and fatty acids thus acquired are, respectively, used to synthesize new proteins and oxidized by mitochondria to produce ATP. Mechanistically, there are two different modes of autophagy in yeast. One is microautophagy, which involves the direct transfer of cytoplasm into the vacuole via invaginations of the vacuolar membrane. The other is macroautophagy, which involves the *de novo* formation of double-membrane vesicles called autophagosomes. Autophagosomes encapsulate cytoplasm and then fuse with the vacuole. Both forms of autophagy are regulated by TORC1 (De Virgilio and Loewith 2006) although, mechanistically, macroautophagy is better understood (reviewed in Cebollero and Reggiori 2009; Nakatogawa *et al.* 2009; Inoue and Klionsky 2010).

Autophagy is conserved across eukarya, and there is much interest in understanding how macroautophagy is regulated as it has been linked to several pathologies including cancer, neurological disorders, and longevity (Yang and Klionsky 2010). In yeast, many **au**toph**ag**y-related (*ATG*) genes encode proteins that participate in the induction of autophagy, the nucleation of the autophagosome, elongation and completion of the autophagosome, and, finally, in fusion of the autophagosome with the vacuole to release the autolysosome into the vacuolar lumen (Chen and Klionsky 2011; Reiter *et al.* 2011). TORC1 regulates macroautophagy by signaling to the Atg1 kinase complex that is required for the induction of macroautophagy. Specifically, when TORC1 is active, Atg13 is hyperphosphorylated, presumably directly by TORC1 (although Tap42-PPase has also been implicated in this regulation), and this prevents the association of Atg13 with Atg1, Atg17, Atg31, and Atg29 (Yorimitsu *et al.* 2009; Kamada *et al.* 2010). Inhibition of TORC1 results in dephosphorylation of Atg13, assembly of the Atg1 protein kinase complex, phosphorylation and

activation of Atg1 (Kijanska *et al.* 2010; Yeh *et al.* 2010), and, subsequently, macroautophagy mediated by as-yet-unidentified Atg1 substrates. Although metazoan homologs exist for many of the Atg1 kinase complex components, a unifying model of how TORC1 regulates this complex in different species has yet to emerge (Chen and Klionsky 2011; Reiter *et al.* 2011).

Cell-wall integrity pathway: The cell wall is essential for yeast cells to survive hostile environments and, more importantly, to prevent internal turgor pressure from rupturing the plasma membrane. Although a thickening of the cell wall helps protect stressed or stationary-phase cells, this rigid structure must also be remodelled to accommodate cell growth. Homeostasis of this structure is maintained by the cell-wall integrity (CWI) pathway (Levin 2005). Cell-wall integrity is monitored by WSC (cell-wall integrity and stress response component) family proteins. WSCs, which are integral plasma membrane proteins, function upstream of the Rho1 GTPase by modulating the activity of the GEFs Rom1 and Rom2. $Rho1^{GTP}$ has several effectors including the yeast protein kinase C homolog, Pkc1. The best-characterized Pkc1 effector is a mitogen-activated protein kinase (MAPK) cascade composed of Bck1 (a MAPKKK), Mkk1 and -2 (redundant MAPKKs), and Slt2/Mpk1 (a MAPK). Activation of this pathway leads to the expression of many cell-wall biosynthetic enzymes, which helps to remodel the cell wall both during normal growth and in response to stress.

Both TORC1 and TORC2 (discussed below) appear to impinge upon the CWI pathway. Entry into stationary phase, carbon starvation, nitrogen starvation, and rapamycin treatment all elicit activation of the CWI pathway, demonstrating that TORC1 negatively regulates the CWI pathway (Ai *et al.* 2002; Krause and Gray 2002; Torres *et al.* 2002; Reinke *et al.* 2004; Araki *et al.* 2005; Soulard *et al.* 2010). Furthermore, *pkc1*, *bck1*, and *mpk1* mutants rapidly lose viability upon carbon or nitrogen starvation, demonstrating that the CWI pathway is required for viability in G0. Mechanistically, how TORC1 signals impinge on the CWI pathway is not clear. Soulard *et al.* (2010) have implicated the Sch9 effector branch while Torres *et al.* (2002) have postulated that signals through the Tap42-PPase branch causes membrane stress that, via WSC family members, activates downstream components of the CWI pathway.

TORC1 accelerates aging: Arguably one of the most interesting functions of TORC1 is its involvement in the regulation of life span. It is well established that, in virtually every biological system, aging, *i.e.*, the progressive deterioration of cell, tissue, and organ function, can be delayed through calorie or dietary restriction. Epistasis studies have led many to believe that this is due to reduced TORC1 signaling (reviewed in Weindruch and Walford 1988; Kapahi *et al.* 2010; Zoncu *et al.* 2010, 2011; Kaeberlein and Kennedy 2011). Indeed, genetic or chemical targeting of TORC1 has been demonstrated to increase life span in yeast, worms, flies, and mice (Vellai *et al.* 2003; Jia *et al.* 2004; Kapahi *et al.* 2004; Wanke *et al.* 2008; Harrison *et al.* 2009; Bjedov *et al.* 2010). These observations have created much excitement in that aging is now thought of as a disease, which, like other diseases, can be ameliorated through pharmaceutical intervention. These observations have also raised the important question, what are the downstream function(s) of TORC1 that modulate life span? The answer to this question is presently unclear, and it is very likely that multiple TORC1 effector pathways contribute (Blagosklonny and Hall 2009). Studies in many model systems are presently underway to address this issue. Below are some of the highlights from studies in yeast.

Yeast life span is assayed in one of two ways. Replicative life span (RLS) is a measure of the number of progeny that a single mother cell can produce before senescence. Chronological life span (CLS) is a measure of the length of time a population of yeast cells can remain in stationary phase before they lose the ability to restart growth following reinoculation into fresh media. RLS is thought to be a paradigm for aging of mitotic cells while CLS is thought to be a paradigm for aging of quiescent cells. Consistent with bigger eukaryotes, where newborns are obviously born young, gametogenesis (*i.e.*, cells derived from meiotic cell divisions) resets RLS in yeast (Unal *et al.* 2011).

Kaeberlein *et al.* (2005) have recently attempted labor-intensive approaches to identify genes involved in both replicative and chronological life span. A random screen of 564 yeast strains, each lacking a single nonessential gene, implicated both *TOR1* and *SCH9* in RLS downstream of caloric restriction. Also identified in this screen were a number of genes encoding ribosomal proteins. Further analyses of *RP* genes subsequently demonstrated that specific depletion of 60S ribosomal protein subunits extends RLS (Steffen *et al.* 2008). Curiously, RLS extension observed upon TORC1 inhibition and 60S subunit depletion seems to be mediated by Gcn4, the TORC1-dependent transcription factor that regulates the expression of amino acid biosynthetic genes as described above. The relevant Gcn4 target genes/processes involved in RLS are not yet known, but an interesting candidate could be macroautophagy. Induction of macroautophagy, like TORC1 and Sch9 inhibition, increases both RLS and CLS (Madeo *et al.* 2010a,b; Morselli *et al.* 2011; and see below), and Gcn4 is required for amino acid-starvation-induced macroautophagy (Ecker *et al.* 2010). Furthermore, spermidine, a potent inducer of macroautophagy, potentially via Gcn4 (Teixeira *et al.* 2010), appears to promote longevity not only in yeast but also in several other model organisms (Eisenberg *et al.* 2009). Since TORC1, Sch9, and Gcn4 homologs are found in most eukaryotes, this appears to represent a conserved aging pathway (Kaeberlein and Kennedy 2011).

Sch9 was one of the first genes to be implicated in CLS (Fabrizio *et al.* 2001). A subsequent high-throughput assay involving 4800 viable single-gene yeast mutants further implicated TORC1 in CLS (Powers *et al.* 2006). These and other studies (Wanke *et al.* 2008; Wei *et al.* 2008) provided evidence that reduced TORC1-Sch9-signaling activity promotes life span by increasing the Rim15-dependent expression of environmental

stress-response genes (described above). Later, Burtner *et al.* (2009) demonstrated that acetic acid-induced mortality is the primary mechanism of chronological aging in yeast under standard conditions and that this toxicity is better tolerated when environmental stress-response genes are artificially induced, for example, upon inhibition of TORC1 or Sch9 activities. However, this model is not universally accepted. Pan *et al.* (2011) have proposed that TORC1 inhibition leads to increased mitochondrial function and a consequent increase in reactive oxygen species that elicit a Rim15-independent pro-survival signal. Furthermore, acetic acid accumulation appears not to be a contributing factor in CLS in this study. Given its apparent conservation across eukarya (Blagosklonny and Hall 2009), elucidation of the mechanisms by which TORC1 regulates life span is eagerly awaited.

Less-characterized effectors identified in phosphoproteomic studies: As alluded to above, large-scale mass spectrometry-based phosphoproteomic studies have recently been performed to identify the rapamycin-sensitive phosphoproteome (Huber *et al.* 2009; Soulard *et al.* 2010). The major limitation of these studies was their poor coverage as evidenced by their rather modest overlap, although this could be partly explained by the different growth conditions and technical approaches employed. Rapamycin exposure times were chosen such that layers of signaling events (*e.g.*, kinase/phosphatase cascades) would be observed. These events should have been triggered as a direct consequence of TORC1 inhibition and not as a secondary consequence of cell cycle delays or changes in transcription. Hundreds of rapamycin-sensitive phosphorylation sites were mapped, the majority of which are in proteins not previously implicated in TORC1 signaling. However, as sufficient time elapsed to activate entire signaling cascades, a potential TORC1 consensus target motif was not evident from the data analyses. Still, the data from these studies will be instrumental in both elucidating how TORC1 signals to its known distal readouts and discovering new TORC1 functions.

TOR Complex 2

Composition and localization of TOR complex 2

TOR complex 2 (TORC2) is rapamycin insensitive and consists of TOR2, Avo1, Avo2, Avo3, Bit61 (and/or its paralog Bit2), and Lst8 (Loewith *et al.* 2002; Wedaman *et al.* 2003; Reinke *et al.* 2004; Zinzalla *et al.* 2010) (Figure 1C). The names of mammalian and invertebrate orthologs of TORC2 subunits and the salient features of *S. cerevisiae* TORC2 subunits are summarized in Table 1 and Table 4, respectively. The highly conserved, essential core subunits are TOR2, Avo1, Avo3, and Lst8. Avo1 and Avo3 bind cooperatively to the N-terminal HEAT repeat region in TOR2 and are required for TORC2 integrity (Wullschleger *et al.* 2005). TORC2 autophosphorylates sites in Avo1 and Avo3, but the purpose of this phosphorylation is unknown. Avo1 has a C-terminal PH-like domain that mediates binding to the plasma membrane (Berchtold and Walther 2009). Avo3 has a RasGEFN domain, a subdomain often found in the N-terminal part of a larger GDP/GTP exchange domain of exchange factors for Ras-like GTPases, but the function of the RasGEFN domain is unknown. Lst8 binds to the kinase domain in TOR2 and is required for TOR2 kinase activity (Wullschleger *et al.* 2005). Lst8 is a Gβ-like propeller protein consisting of seven WD40 motifs. TORC2 is rapamycin insensitive whereas TORC1 is rapamycin sensitive because FKBP-rapamycin binds only TORC1 (Loewith *et al.* 2002). This selective FKBP-rapamycin binding is presumably due to Avo1 masking the FRB domain in TOR2 in TORC2. Finally, co-immunoprecipitation and gel filtration experiments suggest that TORC2 is a multimer, likely a TORC2-TORC2 dimer (Wullschleger *et al.* 2005).

The cellular localization of TORC2 has been studied by subcellular fractionation, indirect immunofluorescence, immunogold electron microscopy, and visualization of GFP-tagged TORC2 components (Kunz *et al.* 2000; Wedaman *et al.* 2003; Aronova *et al.* 2007; Sturgill *et al.* 2008; Berchtold and Walther 2009). In considering these studies, it is important to realize that the vast majority of TOR2 (~90%) is in TORC2 (*vs.* TORC1), and thus TOR2 localization studies presumably detect mainly, if not exclusively, TORC2. All studies indicate that TORC2 is at or near the plasma membrane. Berchtold and Walther (2009) suggest that TORC2 is dynamically localized to a previously unrecognized plasma membrane domain termed the MCT (**m**embrane **c**ompartment containing **T**ORC2). Furthermore, they conclude that TORC2 plasma membrane localization is essential for viability and is mediated by the C-terminal PH domain in Avo1. Most of the localization studies have found that TORC2 is also at another, ill-defined cellular location(s). For example, Kunz *et al.*

Table 4 Salient features of TORC2 components

Protein	Size	Motifs/domains	Potential function
Tor2	2470 aa	HEAT repeats, FAT domain, FRB domain, kinase domain, and FATC domain	Protein kinase, scaffold
Avo1	1176 aa	PH	Recruit TORC2 to plasma membrane
Avo2	426 aa	None obvious	Unknown
Avo3/Tsc11	1430 aa	RasGEFN	Scaffold
Bit61	543 aa	None obvious	Paralogs with unknown function
Bit2	545 aa	None obvious	Paralogs with unknown function
Lst8	303 aa	7 WD-40 repeats	Stabilize kinase domain

Data for this table were obtained from Cybulski and Hall (2009).

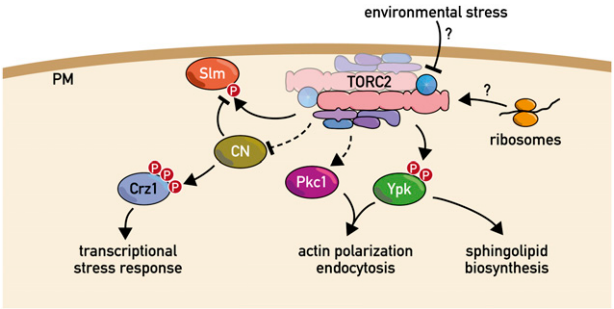

Figure 4 Signaling by TORC2. TORC2 directly phosphorylates the AGC kinase family member Ypk (Ypk1 and 2) and the PH domain containing protein Slm (Slm1 and -2). Downstream effectors include the phosphatase calcineurin, the transcription factor Crz1, and Pkc1. TORC2 controls organization of the actin cytoskeleton, endocytosis, sphingolipid biosynthesis, and stress-related transcription. The effector pathways by which TORC2 controls these processes are incompletely understood (see *Distal readouts downstream of TORC2* for further details).

(2000) report that part of TOR2 is also in an unknown subcellular membrane fraction distinct from Golgi, vacuoles, mitochondria, and the nucleus. Wedaman *et al.* (2003) showed that TOR2 can be in the cell interior often in association with membrane tracks. Sturgill *et al.* (2008) detected a cytoplasmic fluorescent signal in cells expressing GFP-tagged TOR2. In conclusion, TORC2 appears to be at multiple cellular locations, the plasma membrane, and one or possibly more other sites. A plasma membrane location is consistent with the role of TORC2 in controlling the actin cytoskeleton and endocytosis (see below).

Upstream of TORC2

The upstream regulation of TORC2 is poorly characterized (Cybulski and Hall 2009). Several lines of evidence in many different organisms indicate that nutrients regulate TORC1 (see above). On the other hand, there is no reported evidence supporting the notion that TORC2 is controlled by nutrients. Knockout of TORC2 does not confer a starvation-like phenotype, and the nutrient-sensitive EGO complex appears not to be upstream of TORC2. Zinzalla *et al.* (2011) recently devised a "reverse" suppressor screen to identify upstream regulators of TORC2. This screen was based on the observation that constitutively active Ypk2 (Ypk2*) suppresses the loss of viability due to a TORC2 defect. Ypk2 is a protein kinase normally phosphorylated and activated by TORC2 (see below). Zinzalla *et al.* (2011) screened for mutants that require Ypk2* for viability. As predicted, this screen isolated several mutants defective in genes encoding essential TORC2 components, but also in the gene *NIP7*. Subsequent experiments confirmed that Nip7, a ribosome maturation factor, is required for TORC2 kinase activity. The role of Nip7 in the activation of yeast TORC2 has so far not been pursued further, but experiments in mammalian cells suggest that mNip7 is required for mTORC2 activation indirectly via its role in ribosome maturation. In mammalian cells, and presumably also in yeast cells, TORC2 is activated by direct association with the ribosome. As ribosomes determine the growth capacity of a cell, this mechanism ensures that TORC2 is active only in growing cells.

There are also indications that environmental stress inhibits TORC2 signaling, possibly to prevent growth in unfavorable conditions. The mechanism of this regulation and the level at which it intersects with the TORC2 pathway are poorly defined, but it may involve the Slm proteins (see below) and the stress-activated phosphatase calcineurin (Bultynck *et al.* 2006; Mulet *et al.* 2006).

TORC2 substrates

The best-characterized and possibly the major TORC2 substrate is the protein kinase Ypk. Ypk1 and Ypk2 are an essential pair of homologous kinases and members of the AGC kinase family (Roelants *et al.* 2004) (Figure 4). Kamada *et al.* (2005) linked Ypk to TORC2 signaling upon isolating *YPK2* as a multicopy suppressor of a TORC2 defect. They then showed that immunopurified TOR2 directly phosphorylates Ypk2 at Ser641 in the turn motif and Thr659 in the hydrophobic motif. TORC2 phosphorylates and activates Gad8 and SGK1, the *S. pombe* and mammalian orthologs of Ypk, respectively, in a similar manner (Matsuo *et al.* 2003; Garcia-Martinez and Alessi 2008). It is well established that TORC1 or TORC2 phosphorylates the turn and hydrophobic motifs in several kinases as a conserved mechanism of activation of AGC kinase family members (see above) (Jacinto and Lorberg 2008). Ypk/Gad8/SGK1 appears to be a major TORC2 substrate as an *ypk*, *gad8*, or *sgk1* mutation phenocopies a TORC2 defect, and overexpression of Ypk2, Gad8, or SGK1 is sufficient to suppress a TORC2 defect in *S. cerevisiae*, *S. pombe*, or *Caenorhabditis elegans*, respectively (Matsuo *et al.* 2003; Kamada *et al.* 2005; Jones *et al.* 2009; Soukas *et al.* 2009). The two homologous, TORC2- and phosphoinositide ($PI4,5P_2$)-binding proteins Slm1 and Slm2 have also been reported to be phosphorylated in a TORC2-dependent manner both *in vivo* and *in vitro* (Audhya *et al.* 2004; Fadri *et al.* 2005). However, the physiological relevance of Slm phosphorylation is unknown other than that it appears to be required for localization of Slm to the plasma membrane (Audhya *et al.* 2004; Fadri *et al.* 2005).

Distal readouts downstream of TORC2

The first described and best-characterized TORC2 readout is the actin cytoskeleton (Figure 4). TORC2 controls the cell cycle-dependent polarization of the actin cytoskeleton. As the polarized actin cytoskeleton directs the secretory pathway and thereby newly made protein and lipid to the growing daughter bud, this is a mechanism by which TORC2 mediates spatial control of cell growth. The first indication that TOR2 is linked to the actin cytoskeleton came from the isolation of *TCP20*, which encodes an actin-specific chaperone, as a dosage suppressor of a dominant-negative *TOR2* "kinase-dead" mutation (Schmidt *et al.* 1996). This, in turn, led to the discovery that *tor2* mutants display an actin

organization defect (Schmidt *et al.* 1996). The subsequent isolation of *sac7*, which encodes a Rho-GAP (GTPase-activating protein), as a second-site suppressor of a *tor2*-temperature-sensitive (ts) mutation suggested that TOR2 is linked to the actin cytoskeleton via a signaling pathway containing a Rho GTPase. It was later demonstrated that Sac7 is indeed a GAP for Rho1 and that TOR2 activates the Rho1 GTPase switch via the Rho1-GEF Rom2 (Schmidt *et al.* 1997; Bickle *et al.* 1998). Rom2 GEF activity is reduced in extracts from a *tor2*-ts mutant (Schmidt *et al.* 1997; Bickle *et al.* 1998). The finding that overexpression of Rom2 suppresses a *tor2*-ts mutation, whereas overexpression of catalytically active Rom2 lacking its lipid-binding PH domain does not suppress, suggested that TOR2 signals to Rom2 via the PH domain. It was subsequently shown that TOR2 signals to the actin cytoskeleton mainly, if not exclusively, via the Rho1 effector Pkc1 (protein kinase C) and the Pkc1-controlled cell-wall integrity MAP kinase cascade (Helliwell *et al.* 1998b).

How might TORC2 signal to Rom2 to activate the Rho1 GTPase switch? The PH domain in Rom2 suggests that it may involve a lipid intermediate. This possibility is supported by the observation that overexpression of the PI-4-P 5-kinase Mss4 suppresses a *tor2*-ts mutation (Desrivieres *et al.* 1998; Helliwell *et al.* 1998a) and that $PI4,5P_2$ at the plasma membrane is required to recruit/activate Rom2 (Audhya and Emr 2002). The mechanism by which TORC2 may activate $PI4,5P_2$ signaling or possibly a parallel pathway converging on the cell-wall integrity pathway is unknown, but likely involves the well-established TORC2 substrate Ypk (Roelants *et al.* 2002; Schmelzle *et al.* 2002; Kamada *et al.* 2005; Mulet *et al.* 2006). The phosphoinositide-binding Slm proteins and sphingolipids may also be functionally related to TORC2-mediated control of the actin cytoskeleton (Sun *et al.* 2000; Friant *et al.* 2001; Roelants *et al.* 2002; Audhya *et al.* 2004; Fadri *et al.* 2005; Liu *et al.* 2005; Tabuchi *et al.* 2006; Daquinag *et al.* 2007).

A second downstream process controlled by TORC2 is endocytosis. Efficient internalization of cell-surface components is an important aspect of cell growth control. deHart *et al.* (2003) identified a *tor2* mutation in a screen for mutants defective in ligand-stimulated internalization of a cell-surface receptor. TORC2 appears to control endocytosis via Rho1, Ypk1, and possibly the Slm proteins, but how Rho1, Ypk1, and the Slm proteins are functionally related in mediating TORC2-controlled endocytosis is unknown (deHart *et al.* 2002, 2003; Bultynck *et al.* 2006).

A third TORC2-regulated process is sphingolipid biosynthesis (Powers *et al.* 2010). Sphingolipids serve as essential structural components in lipid bilayers and as signaling molecules. The first indication that TORC2 controls sphingolipid synthesis was the finding that overexpression of *SUR1* suppresses a temperature-sensitive *tor2* mutation (Helliwell *et al.* 1998a). In a parallel study, Beeler *et al.* (1998) reported that a mutation in *TOR2* or *AVO3* (also known as TSC11), or mutations in genes encoding components of the sphingolipid biosynthetic pathway, suppress a *csg2* mutation. Sur1/Csg1 and Csg2 are subunits, probably the catalytic and regulatory subunits, respectively, of mannosylinositol phosphorylceramide synthase that mediates a late step in sphingolipid biosynthesis. The Slm proteins were subsequently also linked to sphingolipid metabolism (Tabuchi *et al.* 2006; Daquinag *et al.* 2007). Most recently, Aronova *et al.* (2008) profiled sphingolipids in a conditional *avo3* mutant and thereby confirmed that TORC2 plays a positive role in sphingolipid biosynthesis. Aronova *et al.* (2008) also investigated the molecular mechanism by which TORC2 controls sphingolipids. They found that TORC2 regulates sphingolipid production via Ypk2 and suggest a model wherein TORC2 signaling is coupled to sphingoid long-chain bases (early intermediates in sphingolipid synthesis) to control Ypk2 and late steps in sphingolipid synthesis. Furthermore, the biosynthetic step controlled by TORC2 and Ypk2 is antagonized by the phosphatase calcineurin that is functionally linked to the Slm proteins (Bultynck *et al.* 2006; Mulet *et al.* 2006; Aronova *et al.* 2008). Another potential target for the regulation of sphingolipid biosynthesis by TOR are the Orm1 and Orm2 proteins. The conserved Orm proteins, identified as a potential risk factor for childhood asthma, form a complex that negatively regulates the first and rate-limiting step in sphingolipid biosynthesis (Breslow *et al.* 2010; Han *et al.* 2010). Both Orm1 and Orm2 are phosphoproteins and at least Orm1 phosphorylation changes upon rapamycin treatment (Huber *et al.* 2009; Soulard *et al.* 2010). Furthermore, loss of Orm2 suppresses a Ypk deficiency (Roelants *et al.* 2002; Schmelzle *et al.* 2002; Kamada *et al.* 2005; Mulet *et al.* 2006). These findings suggest that both TORC1 and TORC2 may control sphingolipid synthesis via Orm proteins.

Future Directions

What is upstream of the two complexes?

How TORC activities are altered in response to environmental cues remains a major void in our understanding of the TOR-signaling network. The TOR complexes are regulated by nutrients, stress, or ribosomes, but the mechanisms by which these inputs are sensed and how this information is transduced, with the notable exceptions discussed above, to ultimately regulate kinase activity remain largely unknown. Genetic screens, such as the reverse suppressor screen described above, should help to further elucidate these signaling pathways. Unlike growth factor-signaling pathways, which are present only in metazoans, nutrient and stress-responsive pathways are found in all eukaryotic cells, and thus their characterization in model organisms would have far-reaching implications.

What is downstream of the TORCs?

The TORCs play a central role in the regulation of cell growth by signaling to a staggering number of distal

downstream processes. Recent phosphoproteomics studies have begun to illuminate the relevant phosphorylation cascades and, in addition, have suggested the existence of novel growth-related effectors downstream of TORC1. Similar studies describing the TORC2-dependent phosphoproteome are eagerly anticipated. Elucidating these downstream signaling events is both academically interesting and medically important; cell growth, like cell birth (division) and cell death, is a fundamental aspect of life, and pathological or pharmaceutical dysregulation of TOR pathways is clinically relevant. For example, unbridled ribosome biogenesis has been strongly implicated in cancer, and the motivation to understand the TORC1 effectors that modulate longevity is obvious. Thus, characterization of TOR pathways in yeast and mammals will identify potentially druggable factors whose targeting could yield therapeutic gain in any of several pathologies.

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